

Creating the *fbiA-fbiB*- Double Null Mutant in *Dictyostelium discoideum*

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Allegheny College

Department of Biology

Creating the *fbiA-fbiB*- Double Null Mutant in *Dictyostelium discoideum*

Presented by:

Theodore Avolio

April, 2017

A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for the Bachelor of Science Degree from Allegheny College

***I hereby recognize and pledge to fulfill my responsibilities as defined in the
Honor Code
and to maintain the integrity of both myself and the College community as a
whole.***

Pledge

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Abstract

Dictyostelium discoideum is a soil-dwelling social slime mold that serves as a remarkably useful model organism for studying signal transduction pathways, cell-type proportioning, and the ubiquitin-proteasome system (UPS). In the UPS, proteins are marked for ubiquitin-mediated degradation; therefore, it is a powerful regulatory pathway. FbxA is a protein that has been identified as a part of an SCF ubiquitin-ligase complex. FbxA likely targets another protein known as FbiA for ubiquitin-mediated proteolysis. In *fbiA*- null mutants, the developing organisms exhibit small prespore cell populations and increased prestalk populations. Thus, FbiA likely promotes the prespore pathway while inhibiting the prestalk pathway. A protein homologous to FbiA has been identified via sequence analysis, and it is suitably called FbiB. Mutants of the *fbiB*- null variety show a less severe phenotype relative to *fbiA*- nulls; however, they are comparable. Because of this less severe phenotype, it is suspected that the two proteins “rescue”, consequently demonstrating functional redundancy. Therefore, it is of interest to create an *fbiA-fbiB*- double null mutant in order to study this relationship. This study took steps toward confirming existing pools of double mutant candidates; however, no conclusive evidence emerged from various PCR analyses to verify genuine double mutants in those pools. As a result, a fresh attempt at creating the *fbiA-fbiB*- double knockout was commenced, which began by transforming Blasticidin sensitive *fbiB*- single null mutants. A PCR analysis of newly created double knockout candidates proved to be extraordinarily promising.

Introduction

Development and Specialization of Cells in Dictyostelium discoideum

Dictyostelium discoideum (*D. discoideum*) is a soil-dwelling social amoeba, and when deprived of sustenance, individual cells aggregate via chemotaxis to centers that emit periodic cyclic adenosine monophosphate (cAMP) signals to form a rudimentary multicellular individual (Bhowmik et al. 2016). *D. discoideum* is an extraordinarily useful model organism for studying development since it can exist as a unicellular organism as well as a multicellular organism. Once the mound stage is obtained (Fig 1, letter C), cell differentiation begins, and the two main specialized cell types of prestalk (pst) and prespore (psp) begin to coexist. A 4:1 prespore-to-prestalk is maintained consistently throughout wild-type development. In fact, this proportioning is maintained over an enormous range of aggregate sizes, as a correctly proportioned fruiting body can be produced from as few as 100 cells to an upper extreme of 100,000 cells (Raper, 1940).

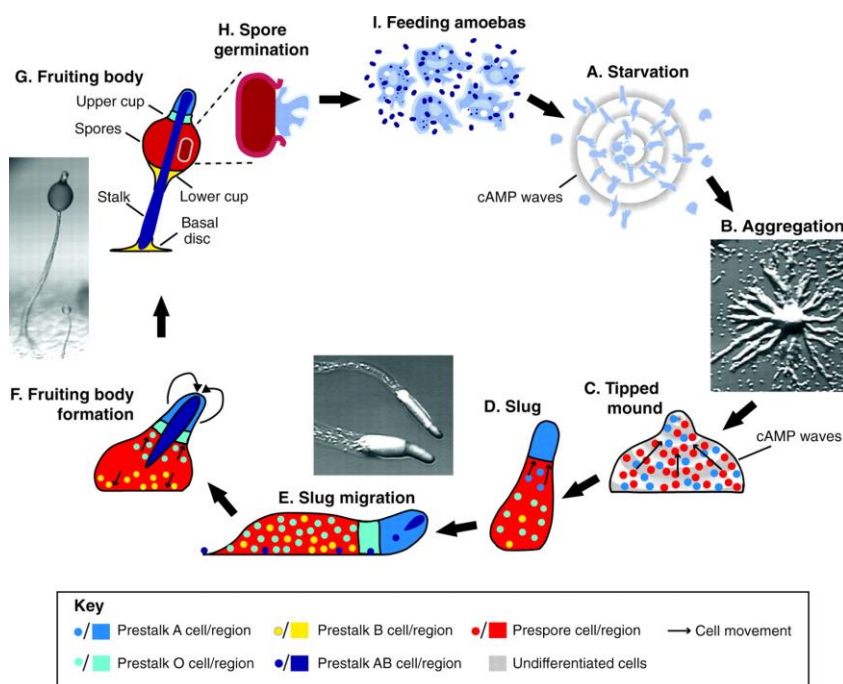


Figure 1. The *Dictyostelium discoideum* life cycle. When starved (A), individual amoebas aggregate (B) to form a tipped mound (C), which then progresses to the migratory slug (D and E), which is a focus for phototaxis and thermotaxis. Eventually, a fruiting body is formed (F and G). (Schaap 2011).

Cells are capable of de-differentiating and re-differentiating between prestalk and prespore types to maintain the correct ratio since as development progresses, as the cells are able to sense surrounding cell types (Early, 1999). As organismal development continues to progress toward the slug stage (Fig. 1, letter D), prespore cells migrate to the posterior region of the slug, while prestalk cells reside in the notably smaller anterior end of the slug. Prestalk cells are further divided into types pstA, pstO, pstB, and pstAB.

The *FbxA* Protein

The *FbxA* protein is prominent when studying signaling pathways in *D. discoideum* because of its prominent role in correct cell type proportioning. Full

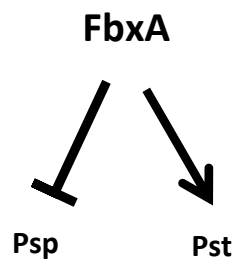


Figure 2. Proposed pathway of of proper *FbxA* functionality.

When *FbxA* is functioning properly, the psp cell population is inhibited, while the pst cell population is promoted.

characterization of the role of *FbxA* in development was done by observing behavior in null mutants and overexpressing mutant varieties. The *fbxA*- null mutants exhibited a smaller prestalk region and a more prominent prespore region, while the *FbxA*++

overexpressor showed a larger prestalk region than the wild-type. In order to see which prestalk populations in *fbxA*- null mutants are aberrant, a reporter construct that is expressed in pstO cells was utilized. It was revealed that there was a

decrease in the pstO population compared to the wild-type, while pstA (analyzed with a different marker) appeared to be unaltered (Nelson et al. 2000). This proportioning of prestalk cells suggests that *fbxA*- nulls have a higher prespore population at the expense of the pstO population.

The FbxA protein contains an F-box protein and WD40 repeats. F-box proteins are a portion of the Skp1, cullin, F-box proteins (SCF) complex; these proteins function to bring target proteins into contact with ubiquitin machinery (Ennis et al. 2000). It has been observed that proteins with the F-box motif bind to the SCF complex (Bai et al. 1996). The WD40 repeat is so named because it is a structural motif containing 40 repeating amino acids which concludes with tryptophan (W) and aspartic acid (D) (Neer et al., 1994). There are several organisms that have proteins that are homologous to FbxA and possess an F-box and WD-40 repeat domain. Organisms like *Caenorhabditis elegans* (Hubbard et al., 1997) and *Drosophila* (Jiang and Struhl, 1998) possess proteins of this type. When faulty, proteins that are functionally similar to FbxA in humans have been linked to neurodegenerative diseases like Parkinson's, Alzheimer's, and Huntington's (Ciechanover and Brundin, 2003). Given the importance to organismal development, there is a great deal of interest in target proteins that interact with FbxA.

F-box Interacting Proteins—FbiA and FbiB

Past comprehensive projects have successfully identified the existence of such a target protein that was aptly given the name “F-box interacting” protein, or FbiA. The existence of this protein was suspected since FbiA homologues had been identified in the aforementioned *C. elegans*, *Drosophila*, and other organisms like *Saccharomyces cerevisiae* (Moré, 2000; Christman et al. 1999), which is a yeast. The present issue is that these homologues of the FbiA protein are not very well understood in terms of their functionality. An *fbiA*- null mutant was successfully created in an attempt to describe the possible role of the FbiA protein. The data illustrated that there was an

increased stalk cell population and a decreased spore cell population compared to the wild-type (McFeaters, 2002). This supports the idea that the normally functioning FbiA protein inhibits prestalk cells while promoting prespore cell development, and therefore

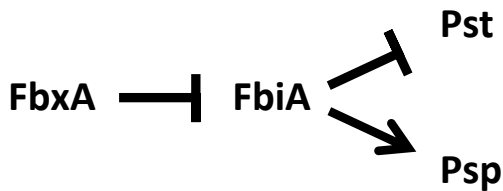


Figure 3. Representation of the proposed pathway of FbiA. FbiA is inhibited by FbxA. FbiA inhibits prestalk cells while promoting prespore cells—therefore, FbxA is a stalk cell promoter.

has a role in cell-type proportioning (Fig. 3).

A homologue of the FbiA protein was identified in *D. discoideum*, and it was appropriately called FbiB. The *fbiB*- null mutants have proved to behave similarly to *fbiA*- null mutants; however, the phenotype of increased stalk cell populations and decreased spore cell populations is not nearly as pronounced (Frick et al. 2011). Consequently, the less prominent phenotype may indicate that even if one of these two F-box interacting proteins is knocked out, then the presence of the other may “rescue” the phenotype to some extent. Therefore, it is ultimately of interest to create an *fbiA-B*-double null mutant in order to investigate whether or not an extremely severe phenotype occurs, which would be indicative of a rescuing property in single null mutants. Additionally, this would further what we know about the role of these target proteins in the FbxA signaling pathway.

Initial steps in *fbiA-fbiB*- creation

A few challenges have presented themselves while trying to create this double null mutant in the past. First is that a new *fbiA*- null mutant had to be created from wild-type that is currently being used in the Nelson lab since the original *fbiA*- null mutant was created via a different wild-type, which is no longer available. The foremost setback occurred in 2012—an *fbiB*- null mutant had been created, and steps were being taken toward the creation of the double null; however, a freezer disaster led to the destruction of much progress. 2015 saw the creation of a new *fbiB*- null mutant, and in fact, an attempt was made at the creation of a double null mutant, but unfortunately, the *fbiA* locus was not disrupted (Tatrn, 2015). Work was done over the course of the summer and fall of 2015 to prepare strains of *fbiB*- nulls that were Blasticidin sensitive so that it could be reinserted with the *fbiA*- disruption cassette to create a double null mutant (Bartlett, 2015). Ultimately, it appears that the double null mutant was not successfully created, as there was no clear disruption at the *fbiA* locus. Consequently, this investigation will commence repeating the *fbiA* disruption in confirmed Blasticidin sensitive *fbiB*- single null mutants.

Hypothesis and Experimental Aims

The first in a series of fundamental goals is to further confirm the differentiation schematic that has been supported by *fbiA*- and *fbiB*- single null mutants, which is that these F-box interacting proteins, when functioning properly, inhibit prestalk

development and promote prespore development. This would be confirmed in a double mutant by observing a severe phenotype where there is an abnormally large/long stalk region and smaller sporehead. However, these observations and analyses can only be performed following the successful creation of the *fbiA-fbiB*- double mutant.

Materials and Methods

Cell Growth Conditions and Maintenance

Cell strains were cultured and stored in tissue culture dishes at 22°C. All cells were grown in axenic medium (1.5% glucose, 0.75% yeast extract, 1.4% bactopectone, 3.6 mM KH₂PO₄, 3.6 mM Na₂HPO₄, 5µg/mL vitamin B12, 0.2 mg/mL Folic Acid), which was changed approximately every 3-4 days. Changing medium consisted of first washing the tissue culture plates with the existing medium, which was then discarded. 10 mL of fresh axenic medium was then pipetted into the plate.

Transformed double mutant candidates were treated similarly, with the exception of the medium mixture. 1 µL of Blasticidin was added per mL of axenic medium and then vortexed to ensure that the Blasticidin was properly incorporated throughout the medium. The final concentration of Blasticidin was 10 µg/mL. This mixture served as the fresh medium for those cell plates.

Genomic DNA Preparation

Small quantities of genomic DNA were required to attempt to confirm genuine *fbiA-fbiB*- double null mutants via polymerase chain reaction. DNA was isolated from ten frozen samples that originated from two different pools of *fbiA-fbiB*- double null mutant candidates, as well as C3 and IIC, “parent and grandparent” strains, respectively,

that were kept in tissue culture dishes. This procedure was also carried out with newly created pools of double knockout candidates called V, W, X, Y, and Z. The Qiagen DNeasy Tissue Kit (Item 69504) was used to perform the DNA isolation, which required 5×10^6 cells from each sample to be centrifuged for five minutes at 300x g—the pellet was then resuspended in 200 μ L PBS (10mM Na_2HPO_4 , 2 mM KH_2PO_4 , 3 mM KCl, 150 mM NaCl). Next, the lysis step was performed by adding 20 μ L Proteinase K and 200 μ L AL Buffer, and samples were incubated at 70° C for ten minutes. Following the incubation step, 200 μ L of 96-100% ethanol was added to each sample to precipitate nucleic acids and then vortexed. The kit provided a DNeasy Spin Column, which each sample was loaded into and was nested in a 2.0 mL collection tube—samples were then centrifuged for one minute at 6000x g. All 12 spin columns were then placed in fresh collection tubes at which point 500 μ L of AW1 Buffer was added to wash the column followed by being centrifuged for one minute at 6000x g. Once again, all spin columns were removed and placed in fresh collection tubes. To wash the membrane, 500 μ L of AW2 Buffer was added and centrifuged at 6000x g. Spin columns were then placed into 1.5 mL microcentrifuge tubes and 200 μ L AE Buffer were applied to the DNeasy membrane was incubated at room temperature for one minute, then centrifuged at 8000x g for one minute to elute the DNA. This elution step was repeated to obtain a second elution in case Elution 1 DNA was overly concentrated. The liquid from all 24 samples (12 “Elution 1”, 12 “Elution 2”), which now contained genomic DNA, was stored in a freezer box until use in polymerase chain reaction. Prior to PCR, however, concentrations of genomic

DNA were checked was a NanoDrop Lite (Thermo Scientific) so that proper dilutions could follow.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed utilizing the genomic DNA acquired via the Qiagen DNeasy Tissue Kit in an effort to ultimately confirm the *fbtA-fbtB*- double null mutant. Master Mix was created by combining 0.2 mM dNTPs, 0.2 μ M forward primer, and 0.2 μ M reverse primer, 1x Advantage Buffer (Clontech), 1x Advantage 2 Polymerase Mix (Clontech) and PCR water. In general, 6.25 μ L template DNA or PCR water (for a DNA absent control) was added into PCR tubes with 3.25 μ L of Master Mix. The PCR was run in an Eppendorf Mastercycler using various programs (Table 1).

Gel Electrophoresis

Gel electrophoresis was the determinant of results from polymerase chain reaction analyses. The gel was composed of 0.8% Agarose (Invitrogen Life Technologies) in 0.5x TAE Buffer (Tris/acetate/EDTA, 50x stock solution: 242g/L Tris base, 57.1ml/L glacial acetic acid, 37.2g/L Na₂EDTA • 2 H₂O). This mixture was microwaved in a standard kitchen microwave in an Erlenmeyer flask until agarose was fully dissolved in buffer. 3.0 μ L of SYBR® Safe (Invitrogen) was added to the solution in the flask. The solution was then allowed to sit and cool for about five minutes so as not to warp the gel mold. After the gel was poured into the mold and allowed to harden for 20 minutes, one well was loaded with 7 μ L of molecular weight marker (Invitrogen), which was a vehicle for comparison. All PCR samples were combined with 1 μ L of blue loading dye

(2.5mg/mL bromophenol blue, 0.4 g/mL sucrose, dH₂O) and then centrifuged to ensure combination and then loaded into remaining wells in the gel. For approximately 50 minutes, a 50-volt electric field was applied to gels in an electrophoresis chamber (LabNet International, Inc.).

Gel Photography

After gels were allowed to run, photographs were taken using a Kodak 1D Imaging System, which utilizes UV light to illuminate the bands. This photography method allowed data to be interpreted visually since bands were to travel specific distances—these distances were indicative of whether or not gene disruptions occurred.

DNA Purification

The QIAquick PCR Purification Kit (Qiagen, 28104). was used in order to purify DNA fragments following PCR reactions used to amplify the disruption fragment. A 5:1 volume ratio of Buffer PB and PCR sample, respectively, were mixed to begin the process. This mixture was then placed in a QIAquick spin column in a 2 mL collection tube provided by the kit. In order to bind the DNA, the sample was centrifuged for one minute at maximum (13,000) rpm. Flow-through was discarded, but the same collection tube was used when 0.75 mL Buffer PE was added to the QIAquick column and centrifuged for one minute 13,000 rpm. Again, flow through was discarded, but the collection tube was reused when the sample was immediately centrifuged again without any new additions to the spin column. However, after this step, the collection tube was discarded and the QIAquick column was placed in a 1.5 mL microcentrifuge tube. For the final elution step, 30 µL Buffer EB (10 mM Tris-Cl) was added to the center of the

QIAquick membrane and allowed to stand for one minute before a concluding centrifuge for one minute at 13,000 rpm. DNA concentration was then checked with a NanoDrop Lite (Thermo Scientific).

Measuring Concentrations of Genomic DNA

Concentrations of genomic DNA and the *fbiA*Δ*Bsr* fragment were measured using a NanoDrop Lite system (Thermo Scientific)—a UV spectrophotometer that is designed specifically for use with microvolumes of purified proteins and nucleic acids. 1.5 μL of each sample of genomic DNA was applied to the instrument's stainless steel pedestal, and the resulting data outputs that were recorded were absorbance (A260), purity ratio (A260/A280), and concentration.

Ethanol Precipitation

Ethanol precipitation was performed in order to further concentrate the PCR product. Since DNA transformations of this type require an approximate concentration of 350 ng/ μL and this threshold had yet to be met. For the total volume of genomic DNA sample, 1/10 of that volume of 3M NaOAc and 2.5x the volume of 100% EtOH was mixed. Two ethanol precipitations were performed, and the first had the solution placed in a -20° C freezer overnight while the second procedure had the solution placed in a -80°C freezer for 20 minutes. To pellet the DNA after freezing, the solution was centrifuged for 10 minutes at 13,000 rpm. The supernatant was removed, and 250 μL of 70% EtOH was added. The pellet was resuspended by gently inverting the tube which was then centrifuged once again at 13,000 rpm. Following the removal of supernatant, the sample was allowed to air dry for 10 minutes. Once the sample was dry, 10 μL of

PCR water was added to the tube, and the concentration of the entire sample was analyzed with the NanoDrop Lite Spectrophotometer.

Testing for Blasticidin Sensitivity

For consistency's sake, all cell densities were diluted to 2×10^5 cells/mL in axenic medium. The cell strain intended for transformation is known as C3, which is a confirmed *fbiB*- null mutant. Transformation requires C3 to be sensitive to Blasticidin (Bs^S), and this was the variable in question. To test this, three cell lines were used: Ax2 (previously confirmed as Bs^S), CII (previously confirmed to be Bs^R), and C3 (the experimental cell line). 5 mL of cells were applied to two 60 mm plates for a total of six, with each cell line having a no antibiotic control plate and a plate in which 5 μ L of Blasticidin was added to the cells for a final Bs concentration of 10 μ g/mL. Cells were maintained in this manner with medium changes every 3-4 days, and regularly checked to ensure that Bs^S Ax2 and the presumed Bs^S C3 cells died, while Bs^R CII cells thrived. C3 was ultimately confirmed as Bs^S in order to meet the requirement for transformation.

Transformations via Electroporation

A disruption fragment was introduced to the cells by utilizing electroporation (procedure adapted from Gaudet, 2007). Prior to electroporation, Bs^S *fbiB*- null cells were grown to a density of $1.0\text{--}2.5 \times 10^6$, as 5×10^6 cells are required for transformation. 10 mL of H50 buffer (20mM HEPES, 50mM KCl, 10mM NaCl, 1mM $MgSO_4$, 5mM $NaHCO_3$, 1mM NaH_2PO_4) and an additional 200 μ L (to be used in transformation) were held on ice. Using the IEC Centra GP8R Refrigerated Centrifuge (Thermo Electron

Corporation), a pellet was formed by centrifuging the cells at 2000 rpm for 4 minutes. The supernatant was poured off, 5mL of the now cold H50 buffer was added. Those steps to harvest and wash cells were repeated. After cells were pelleted, the supernatant was disposed of and cells were diluted into a 5×10^7 cells/ mL in solution with H50. Next, 100 μ L of cells were transferred into a chilled Eppendorf tube, and 10 μ L of amplified DNA plasmid was added, while a no DNA control remained untouched. This cell+DNA solution was transferred into chilled electroporation cuvettes, and electroporation at a pulse of 0.7kV, 25 μ FD with a Gene Pulser (Bio-Rad)—there were two pulses separated by 5 seconds. Following electroporation, cells rested on ice for 5 minutes, at which point they were transferred into tissue culture dishes.

Before electroporation was performed, 10 mL of axenic medium was added to a 90 mm TC dish for the no DNA control. 10 mL of medium was added to a sterile 15 mL tube for the +DNA transformants. A kit-provided plastic pipette was used to transfer cells into either the TC plate or tube. To ensure maximum cell recovery, the cuvette was washed with medium repeatedly. The cell+medium mixture of the +DNA transformation was split among five wells of a six-well plate with 2 mL per well. Cells were incubated at 22° C.

The following day, heat-killed bacteria and Blasticidin (selective agent) were added to the cells. Medium with Blasticidin was used to change medium without washing every 3-4 days until colonies developed in the +DNA plates.

Plating for Clonal Isolates on Klebsiella

Prior to plating, two SM agar plates (1% glucose, 4mM MgSO₄, 14mM

KH₂PO₄, 5.7 mM K₂HPO₄, 1% Bacto-peptone, 1% yeast extract, 2% agar) were poured per pool. A lawn of *Klebsiella* was also plated in advance on the SM agar and was incubated at 37° C. A suspension of the bacteria was formed in sterile KK2 (16.5 mM KH₂PO₄, 3.8 mM K₂HPO₄) using a few loopfuls of cells from the bacterial lawn. *D. discoideum* cells from Pools 1 and 3 were diluted to 1x10³ cell/mL. Next, 100 µl of the bacterial suspension was placed onto each SM agar plate in conjunction with the diluted *D. discoideum* in order to place 50-100 cells on each plate. Cells were spread on the plate with a sterilized stainless steel spreader. To avoid letting the plates dry out, parafilm was wrapped around the plates' edges, and then plates were incubated at 22° C. The bacterial lawn grew within 24 hours, and *D. discoideum* plaques appeared 5-7 days after.

To remove clonal isolates, axenic medium was placed in ten 60mm petri dishes. A sterile hood designated for bacterial cultures was used when a sterile, wooden applicator stick was used to transfer appropriate colonies into the petri dishes, which were filled with 5 mL of axenic medium. Cells were then allowed to grow and establish themselves, and routine maintenance techniques were performed.

Experimental Templates and Plasmids	Primer Pairs	PCR Conditions	Expected Band Sizes
A - Pools 1, 3, and 5 - Clonal populations from pools 1 and 3 - Pools V, W, X, Y, and Z	544/Bs-Rev	Program: AB_FBI_A_KO 94° C for 3 minutes— <i>denaturing</i> 94° C for 30 seconds— <i>denaturing</i> 54° C for 1 minute— <i>annealing</i> 68° C for 2 minutes— <i>extension</i> 68° C for 10 minutes— <i>extension</i> 4° C holding temperature	Ax2 & C3: $\emptyset$ (intact <i>fbiA</i>) fbiA: 1.6 kb (disrupted <i>fbiA</i>)
B - Pools 1, 3, and 5 - Clonal populations from Pools 1 and 3 - Pools V, W, X, Y, and Z	Pair 1 - Bs-Fwd/R11 Pair 2 544/R11	Program: MAFBIA 94° C for 3 minutes— <i>denaturing</i> 94° C for 30 seconds— <i>denaturing</i> 51° C for 1 minute— <i>annealing</i> 68° C for 2.5 minutes— <i>extension</i> 68° C for 10 minutes— <i>extension</i> 4° C holding temperature	Pair 1 Ax2 & C3: No PCR product (intact <i>fbiA</i>) fbiA: 2.2 kb (disrupted <i>fbiA</i>) Pair 2 Ax2 & C3: 1.8 kb (intact <i>fbiA</i>) fbiA: 3.35 kb (disrupted <i>fbiA</i>)
C - C3 - FbiA	544/R11	Program: TA_GRAD_A 94° C for 3 minutes— <i>denaturing</i> 94° C for 30 second— <i>denaturing</i> (51-56)° C for 1 minute— <i>annealing</i> 68° C for 3.5 minutes— <i>extension</i> 68° C for 10 minutes— <i>extension</i> 4° C holding temperature	C3: 1.8 kb (intact <i>fbiA</i>) fbiA: 3.35 kb (disrupted <i>fbiA</i>)

D - C3 - FbiA	544/R11	Program: TA_GRAD_B 94° C for 3 minutes— <i>denaturing</i> 94° C for 30 seconds— <i>denaturing</i> (56-59)° C for 1 minute— <i>annealing</i> 68° C for 3.5 minutes— <i>extension</i> } 37x 68° C for 10 minutes— <i>extension</i> 4° C holding temperature	C3: 1.8 kb (intact <i>fbiA</i>) fbiA: 3.35 kb (disrupted <i>fbiA</i>)
F Clonal populations from Pools 1 and 3	544/R11	Program: TA_FBIA 94° C for 3 minutes— <i>denaturing</i> 94° C for 30 seconds— <i>denaturing</i> 56° C for 1 minute— <i>annealing</i> 68° C for 3.5 minutes— <i>extension</i> } 37x 68° C for 10 minutes— <i>extension</i> 4° C holding temperature	C3: 1.8 kb (intact <i>fbiA</i>) fbiA: 3.35 kb (disrupted <i>fbiA</i>)
F <i>fbiA</i>∇Bsr	618/2275	Program: TA_BSR_FRAG 94° C for 3 minutes— <i>denaturing</i> 94° C for 30 seconds— <i>denaturing</i> 52° C for 1 minute— <i>annealing</i> 68° C for 3.5 minutes— <i>extension</i> } 37x 68° C for 10 minutes— <i>extension</i> 4° C holding temperature	fbiA: 3.2 kb

Table 1. PCR programs overview. The course of this investigation saw many different experimental templates and plasmids that required various PCR conditions and primers—resulting band lengths were contingent upon the factors of starting template or plasmid and primers pairs.

Results

Overview of Experimental Design

Previously, steps toward the creation of the *fbiA-fbiB*- double null mutant were taken, and this study followed up on that work as well as started an independent path toward the creation of the double knockout. In both cases, cell lines were transformed with a Blasticidin-resistance cassette that serves a twofold purpose. The first is that transformed cells can be selected easily since cells without the disruption fragment will be killed when introduced to Blasticidin. Additionally, the gene is disrupted and consequently produces an improper protein product.

This investigation began by using previously transformed *fbiA-fbiB*- double null candidates and took further action toward confirming the possibility of genuine double knockout mutants (Bartlett, 2015). A PCR analysis was performed on pools of candidate mutants as well as clonal isolates. The purpose of the individual pools was to make certain that mutants developed independently of one another. This is an important design since it is possible that an altered phenotype could arise as a result of a secondary insertion event. Independent pools ensured that if a mutation from a secondary insertion event theoretically occurred, then it would only affect the individual pool rather than the entire population of transformed cells. This safeguard guarantees that phenotypic alterations are the result of a properly disrupted *fbiA* locus rather than some random secondary insertion event. Furthermore, this design allowed for pools that contained no mutant cells to be eliminated by way of PCR. If a pool exhibited a disruption event, clonal isolates were developed from a situation where single cells

were allowed to form colonies. If a subsequent PCR screening presented evidence of a disruption event, then a genuine double mutant would be confirmed since any random disruptions had previously been discredited.

The independent path toward the creation of the *fbiA-fbiB*- double knockout began by transforming a confirmed Bs^S *fbiB*- null mutant known as C3. Being sensitive to Blasticidin allowed C3 to be transformed with the *fbiA* ∇ Bsr plasmid template, which would consequently make a successful double knockout mutant Bs^R .

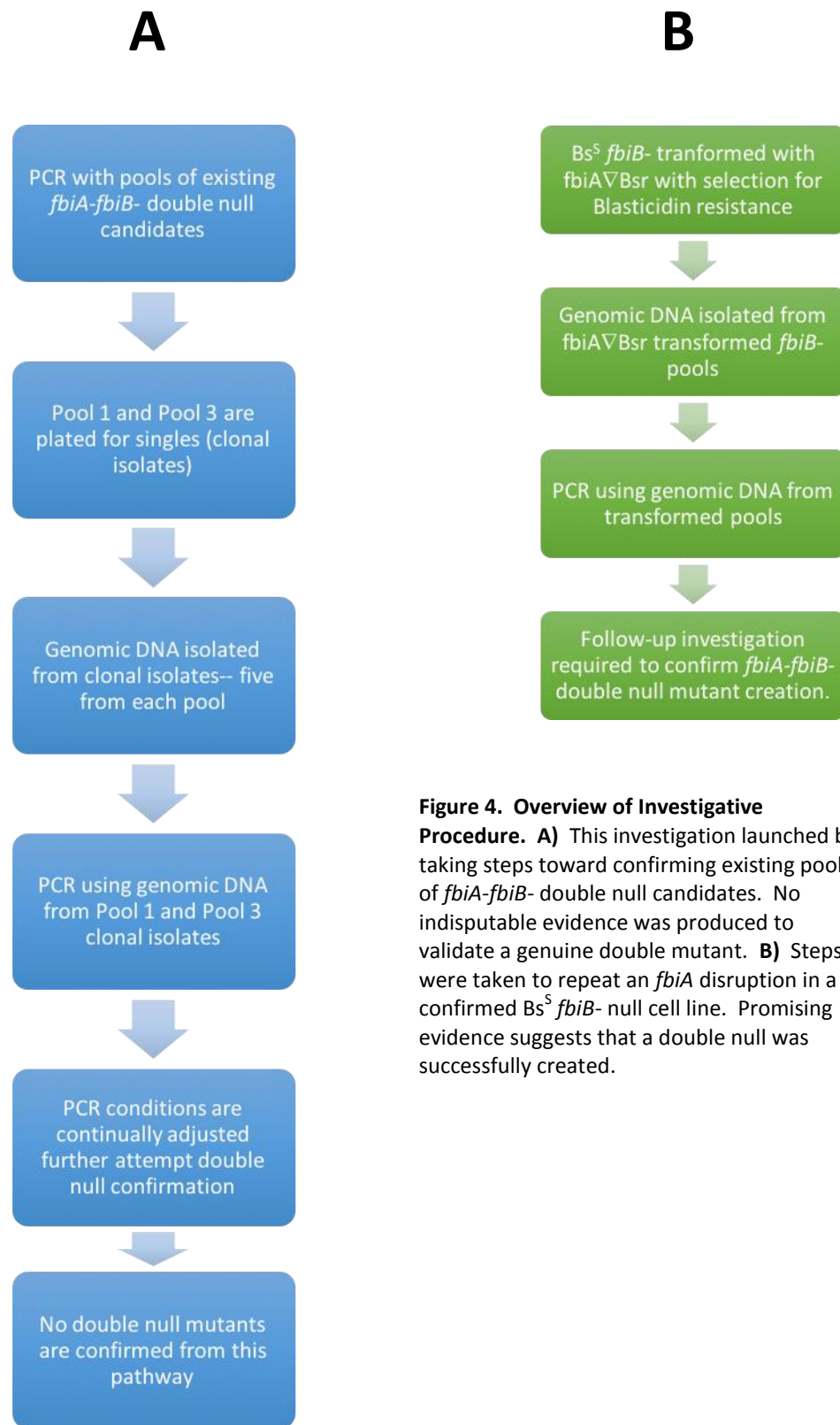


Figure 4. Overview of Investigative Procedure. **A)** This investigation launched by taking steps toward confirming existing pools of *fbiA-fbiB*- double null candidates. No indisputable evidence was produced to validate a genuine double mutant. **B)** Steps were taken to repeat an *fbiA* disruption in a confirmed *Bs^S fbiB*- null cell line. Promising evidence suggests that a double null was successfully created.

PCR Screening for Existing Pools of Double Mutant Candidates

Previous work determined that pools, denoted as “Pool 1”, “Pool 3”, and “Pool 5”, were viable *fbiA-fbiB*- double null candidates. The work in this investigation began with a PCR analysis of said pools in order to further confirm their validity as genuine double knockout mutants. Two PCR analyses were performed using two primer pairs: R11/Bs-Fwd and 544/Bs-Rev (Table 1, A & B). These primer pairs produce different band lengths when gel electrophoresis is performed (Fig. 5). Both PCR analyses also used a DNA null control, a negative control (intact *fbiA* locus), and a positive control (confirmed *fbiA* single null mutant).

The subsequent gel electrophoresis for the R11/Bs-Fwd combination illustrated that Pool 3 was the most likely of the three pools to contain genuine double mutants since a band was present on the gel at 2.2 kb— this length is indicative of a disruption event at the *FbiA* locus (Fig. 6A). Similarly, the gel for the 544/Bs-Rev primer pair produced a band indicative of an *FbiA* locus disruption event at 1.6 kb (Fig. 6B). Figure 6B also shows a fainter band at the proper disruption band length for Pool 1; however, the fainter band and other background in the Pool 1 column made the pool a less appealing double mutant candidate. Additionally, Pool 1 was a less appealing candidate because of the ostensibly missing band at the proper 2.2 kb band length, which both the confirmed *fbiA*- single null positive control and Pool 3 exhibit. Yet, Pool 3 remained unconfirmed as well with the 544/Bs-Rev primer pair since a considerable amount of background was present; thus, the band present at 2.2 kb for Pool 3 could plausibly be mere background as well.

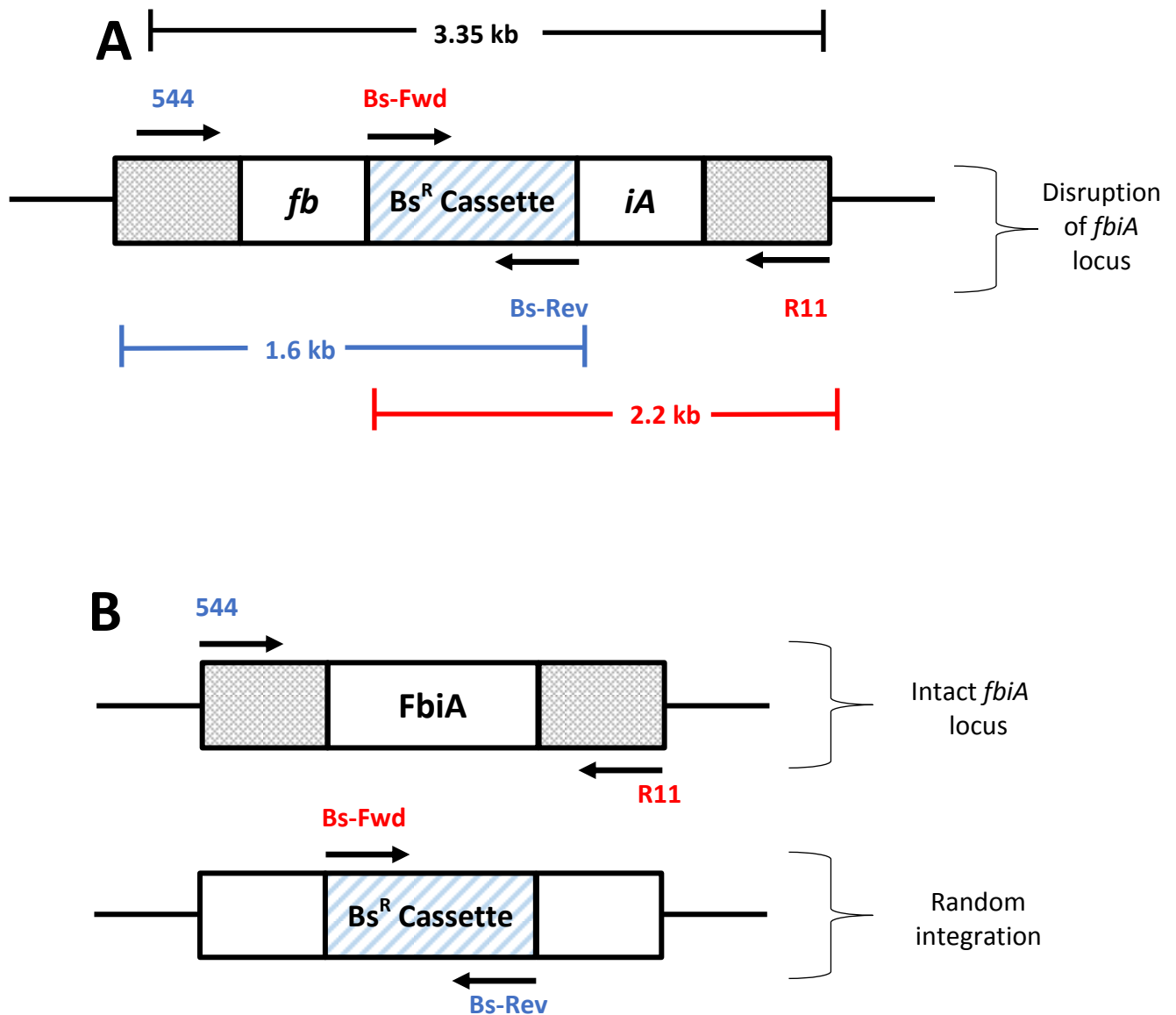


Fig 5. Representation of the *fbiA*- disruption construct. A) The 544/Bs-Rev primer pairing (depicted in blue) produces a band length of about 1.6 kb when a disruption even occurs, while the Bs-Fwd/R11 primer pairing (depicted in red) produces a band that is of approximately 2.2 kb in length. The 3.35 kb band length for the 544/R11 pairing is shown in black. Note: the above representation is not to scale to depict relative lengths. **B)** In the absence of an insertion even with a Blasticidin resistant (Bs^R) cassette, the *fbiA* locus is left intact. The Bs-Fwd and Bs-Rev primers have no template to bind to in this scenario since they are both located on the Bs^R cassette, so no band is produced with an intact *FbiA* locus, and therefore, no PCR product is produced. This outcome also indicates that random integration of the Bs^R cassette occurred

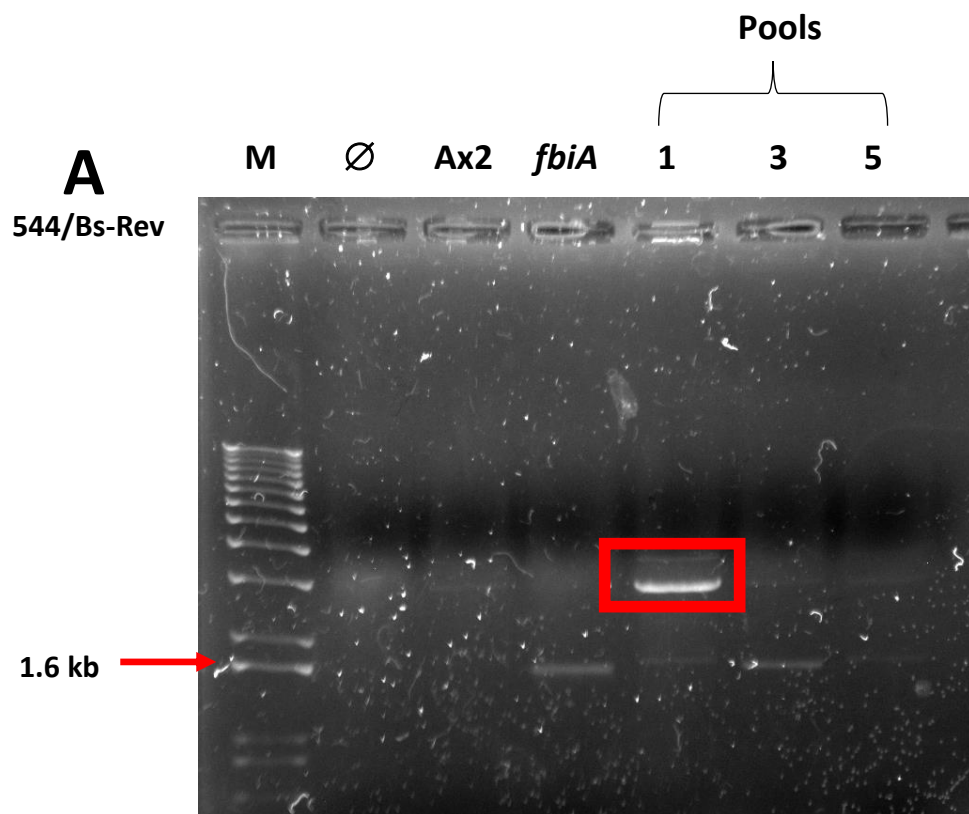
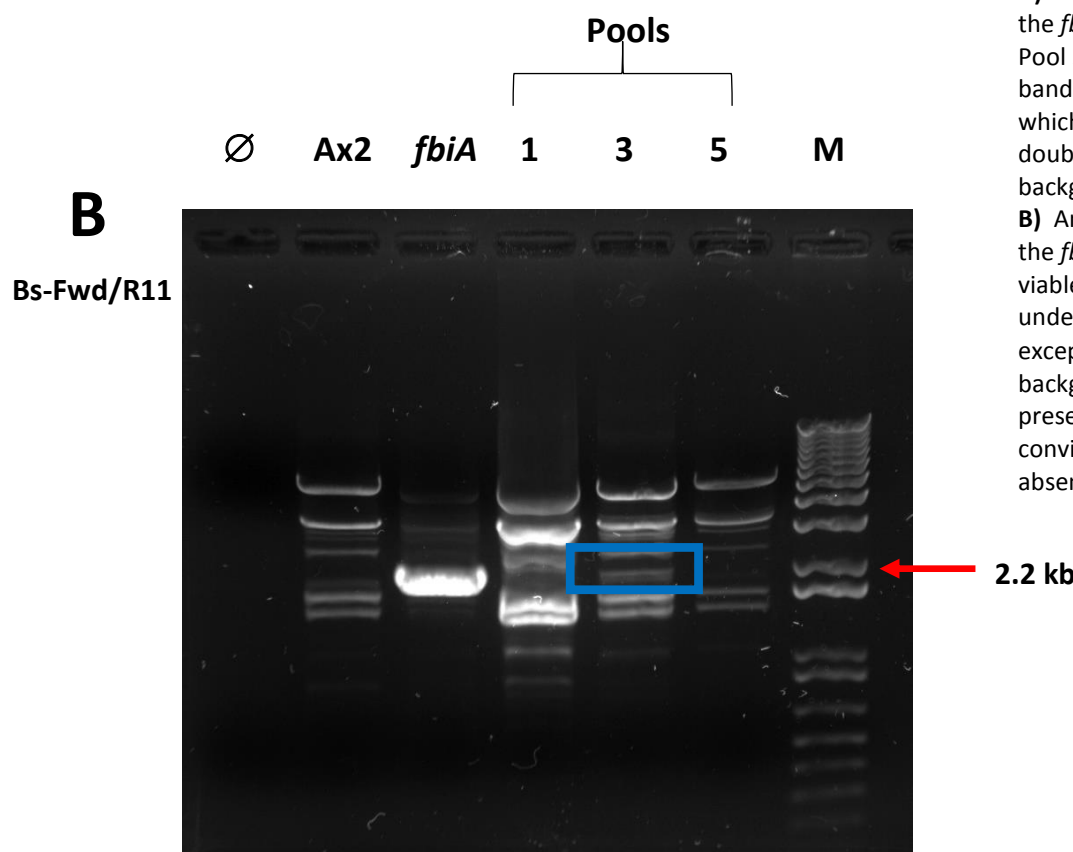


Figure 6. Gel electrophoresis of existing double knockout candidates.

Pools of *fbiA-fbiB*- double mutant candidates and their associated controls ran on a 0.8% agarose gel via electrophoresis. 7 μ L of molecular weight marker was added to the lane labeled “M”. 10 μ L of template/loading dye mixture was added to all other lanes.

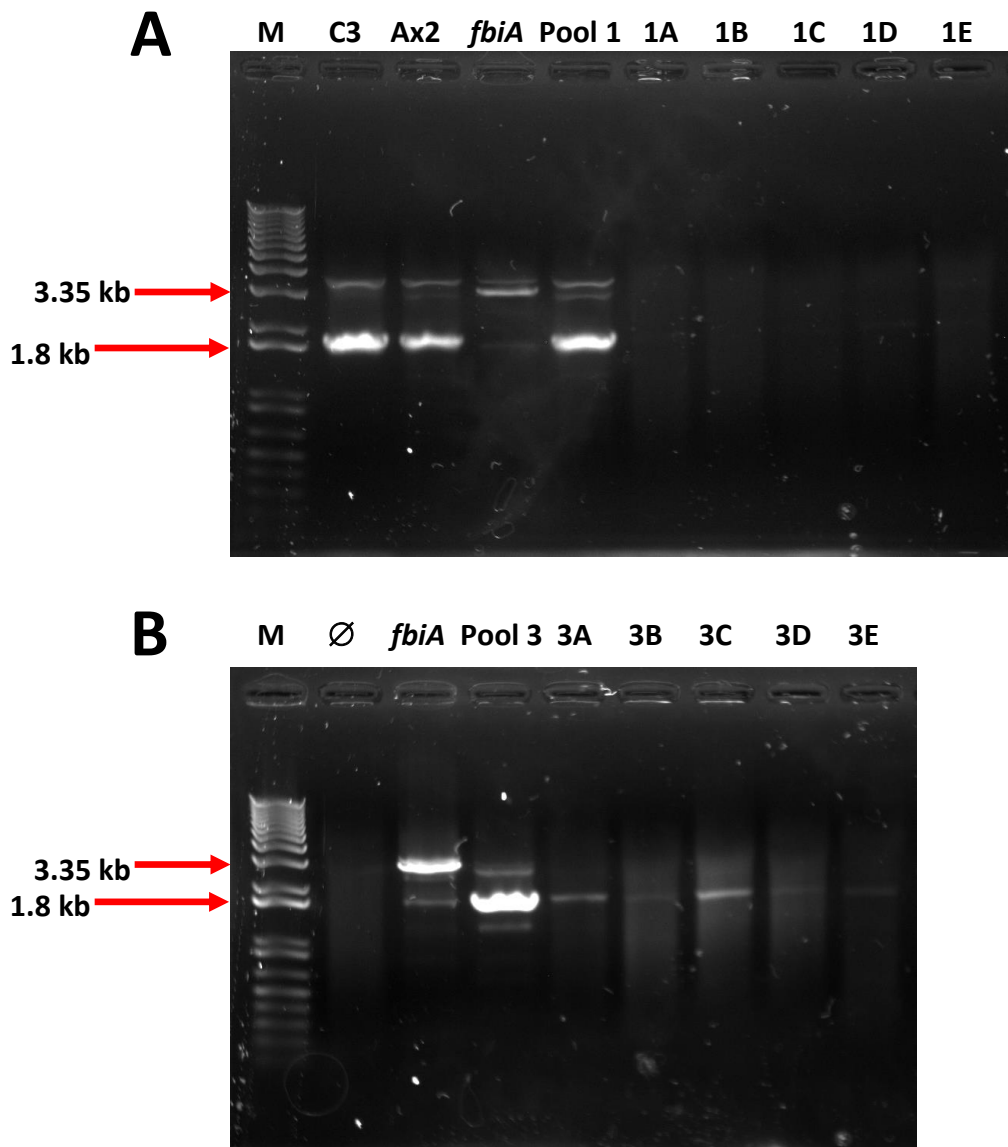
A) Expected bands are visible at 1.6 kb for the *fbiA* and Pool 3 lanes, further advancing Pool 3 double mutant candidacy. A faint band is seen in Pool 1 at the 1.6 kb length, which provides a less convincing chance for double mutant candidacy. A stray background band is boxed in red.

B) An expected band is present at 2.2 kb in the *fbiA* positive control lane. A potentially viable *fbiA* disruption band is boxed in blue under Pool 3; however all lanes with the exception of Ø and *fbiA* are riddled with background noise. Consequently, the band present in Pool 3 at 2.2 kb is far less convincing. Pool 1 and Pool 5 exhibit an absence of a band at 2.2 kb.



Clonal Isolates from Existing Mutant Candidates Undergo PCR Analysis

Pool 1 and Pool 3 maintained varying degrees of potential to be confirmed as double mutants following the initial PCR screening of Pools 1, 3, and 5 with Pool 3 serving as the leading candidate. Therefore, five clonal populations from each of both Pool 1 and Pool 3 were cultured on *Klebsiella* lawns for a total of ten clonal populations (denoted as 1A-1E and 3A-3E). Genomic DNA from these populations was alongside Bs^S “C3” and Bs^R “IIC”.



A PCR analysis was performed using the clonal isolates from Pools 1 and 3 using the 544/R11 primer pairing. No convincing evidence of genuine double null mutants was displayed by any of the clonal isolates. The Pool 1 clonal isolates failed to produce any bands—even a 1.8 kb band indicative of an intact *fbiA* locus (Fig. 7A). Pool 1 and *fbiA* both yielded the 3.35 kb band that is expected from the disruption event; however, Pool 1 also produced a sizeable 1.8 kb band. Modifications of PCR conditions were implemented (data not shown), but similar results were produced and no notably convincing evidence of a double null mutant was uncovered from Pool 1.

All Pool 3 clonal isolates displayed faint 1.8 kb bands, but the *fbiA* control also produced a faint band at that length; thus, those faint bands are conceivably just background noise. The Pool 3 lane presented an ambiguous result with a heavy 1.8 kb band and a faint 3.35 kb band, so nothing conclusive was exhibited from Pool 3 in this analysis. Pool 3 and its clonal isolates were subjected to the PCR conditions modifications as well, and much like Pool 1 and its clonal isolates, no evidence arose suggesting a successful double null mutant.

Preparing *fbiA* ∇Bsr Disruption Fragment

Transformation of *Bs^s fbiB-* null C3 required the *fbiA*∇Bsr plasmid that contains a disruption fragment. This plasmid was amplified via PCR with the 618/2275 primer pairing, for which the expected band length is 3.2 kb when confirmed with electrophoresis (Fig. 8). Two trials were necessary because the first trial did not produce the target concentration of approximately 400 ng/μL of purified DNA fragments

that are required for transformation. The second trial fell short of the target concentration as well following purification. An ethanol precipitation was performed following both trials, but concentrations remained still too low. The solutions of amplified plasmids were combined to finally reach a concentration exceeding 400 ng/μL.

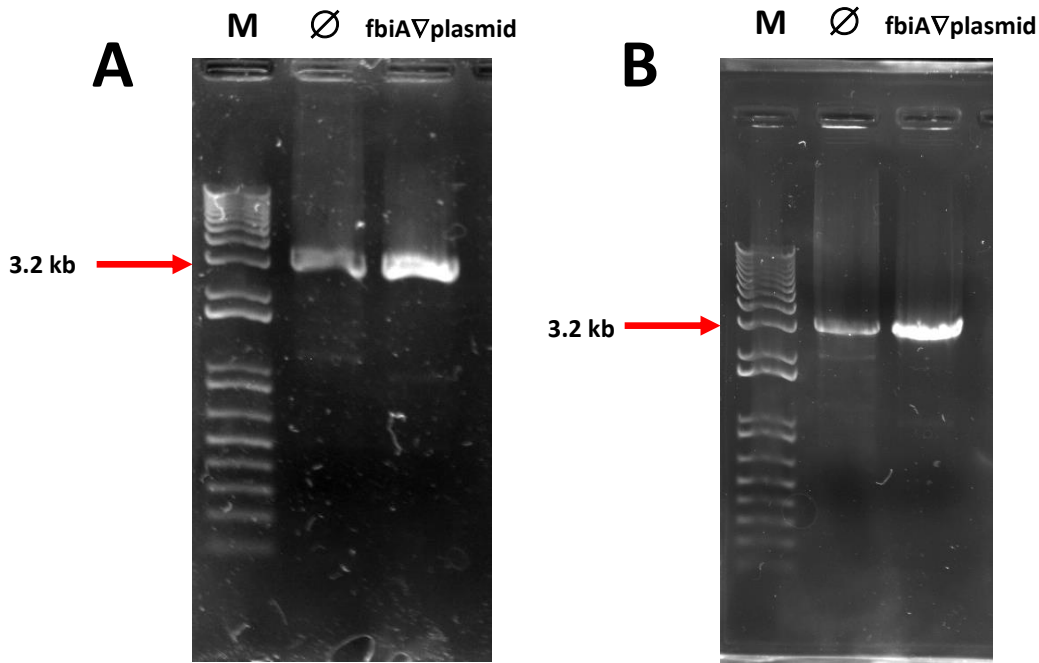


Figure 8. Confirming amplification of the fbiA ∇ Bsr plasmid. Two plasmid amplifications were required since the concentration of each alone was never at the target 400 ng/μL. Both trials **A** and **B** produced 3.2 kb bands in the plasmid lane. The 3.2 kb band in the null DNA lanes in both trials can be ignored as harmless background.

Screening for Newly Transformed Pools

Following transformation via electroporation, five pools denoted as V, W, X, Y, and Z underwent PCR analysis in order to confirm *fbiA-fbiB*- double null candidacy. This parallels the results in Figure 6B, as the Bs-Fwd/R11 primer pairing was utilized once again; therefore, the expected band length was 2.2 kb. However, the resulting data of these newly transformed pools was remarkably more conclusive than the older pools.

With virtually no background bands, all pools with the exception of Pool V produced a the 2.2 kb band that is indicative of a successful disruption at the *fbiA* locus.

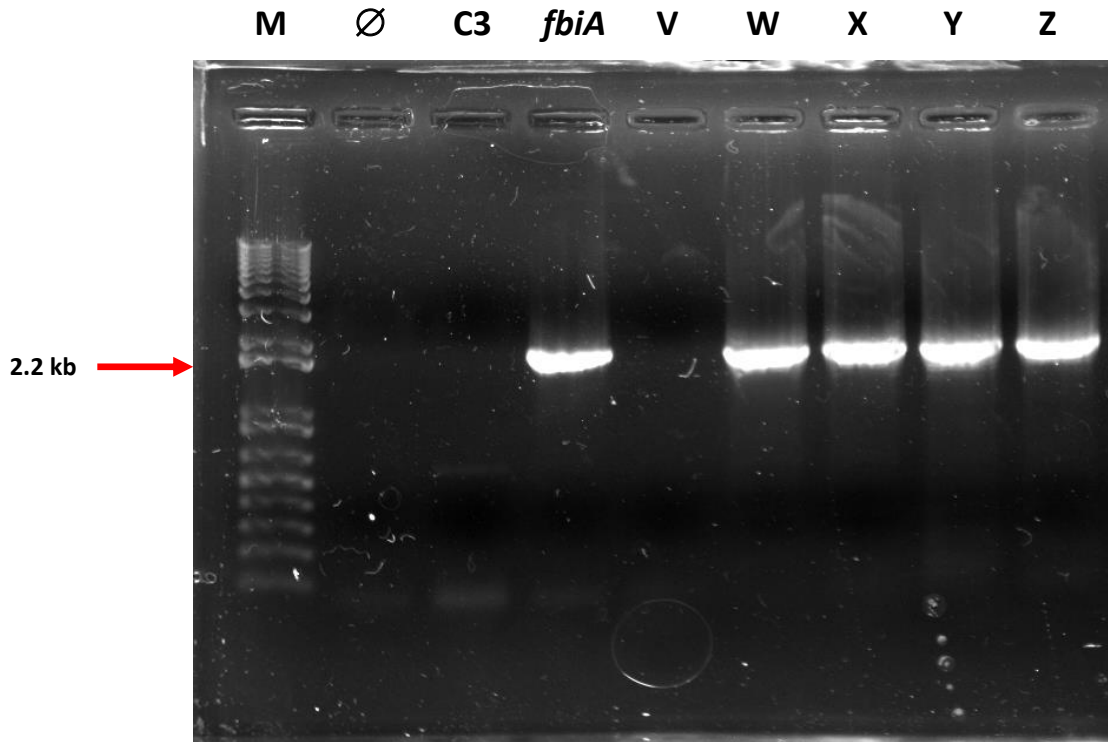


Figure 9. Confirming double null candidacy in transformed pools. All pools with the exception of V produced the 2.2 kb band that is indicative of a disruption at the *fbiA* locus. The *fbiA*- positive control properly produced this band as well. The C3 and DNA null slots appropriately displayed blank lanes, as is characteristic of an intact *fbiA* locus.

Discussion

Current Standing

This study established two principle objectives. The first intent was confirming existing *fbiA-fbiB*- double null mutants in order to study possible functional redundancy between FbiA and FbiB; however, when existing candidates failed to produce conclusive evidence of a disruption at the *fbiA* locus, a fresh attempt at the creation of a double null mutant commenced. A PCR analysis and gel electrophoresis of new candidate pools

V, W, X, Y, and Z concluded in a very promising fashion, as all pools except Pool V exhibited overwhelming evidence of a disruption at the *fbiA* locus. Therefore, there is considerable optimism that the double null mutant was successfully created.

Future Directions

The next step for future expansion upon this study is to confirm genuine double mutants with clonal populations that are verified via PCR analysis. Once confirmed, developments can be made in order to observe whether or not a more severe phenotype occurs in the double null mutant relative to the *fbiA*- and *fbiB*- single null mutants. Regardless of whether or not there is functional redundancy in these homologous genes, LacZ reporter transformations can be performed in order to characterize the double mutant. It can be determined how the prespore-to-prestalk ratio has been altered since single nulls are known to have increased prestalk populations and severely reduced prespore regions (Fig. 10). A potential direction for these transformations is to use LacZ reporters PspA-gal and EcmO-gal, which mark prespore and prestalk O cells, respectively. An EcmO-gal reporter, specifically, is appropriate since an analysis of various FbxA mutants illustrated that prestalk O is the type of prestalk cell that is generally the most affected (McFeaters, 2002).

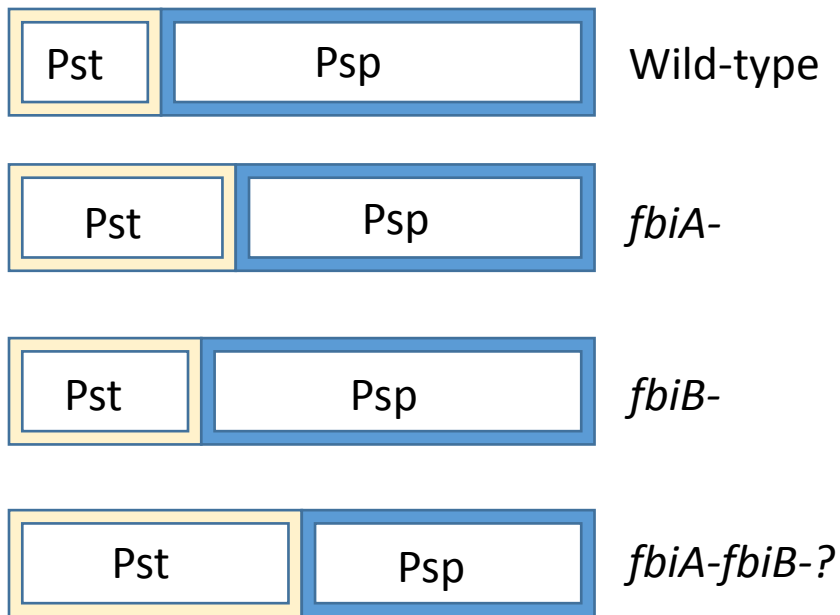


Fig 10. Depiction of Pst:Psp Ratio in Various *D. discoideum* Strains. Proportioning of Pst:Psp cells in wildtype *D. discoideum* is about 1:4. The proportions depicted in each of the strains are arbitrary, but relative. *FbiA*-null mutants show an increased Pst population, as do *fbiB*- null mutants; however, *fbiB*- null mutants are less pronounced in terms of increased prestalk population. The proportioning in the *fbiA-fbiB*- double null mutant is unexplored, but with its successful creation, the proportioning can be examined.

Significance

The ultimate goal has been to further what is known about these target proteins. Homologues of the F-box interacting proteins have been identified in other organisms, but functionality has lacked exploration. Thus, it is valuable to increase the bank of information about these types of target proteins and to learn more about their functionality. Since *D. discoideum* contains many evolutionarily conserved genes, it is important that the powerful relationship between the homologous FbiA and FbiB genes be better understood.

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